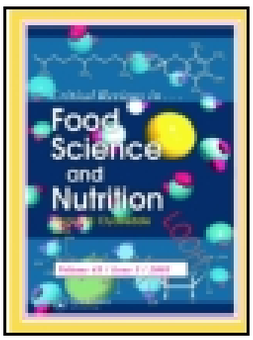

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Effects Of Inorganic Nitrate And Nitrite Consumption On Cognitive Function And Cerebral Blood Flow: A Systematic Review And Meta-Analysis Of Randomised Clinical Trials

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Abstract

We conducted a systematic review and meta-analysis of randomized clinical trials examining the effect of inorganic nitrate or nitrite supplementation on cognitive function (CF) and cerebral blood flow (CBF). Two databases (PubMed, Embase) were searched for articles from inception until May 2017. Inclusion criteria were: randomized clinical trials; participants >18 years old; trials comparing a nitrate/nitrite intervention with a control. Thirteen and nine trials were included in the meta-analysis to assess CF and CBF, respectively. Random-effects models were used and the effect size described as standardized mean differences (SMDs). A total of 297 participants (median of 23 per trial) were included for CF; 163 participants (median of 16 per trial) were included for CBF. Nitrate/nitrite supplementation did not influence CF (SMD +0.06, 95% CI: -0.06, 0.18, P =0.32) or CBF under resting (SMD +0.14, 95% CI: -0.13, 0.41, P =0.31), or stimulated conditions (SMD +0.23, 95% CI: -0.11, 0.56, P =0.19). The meta-regression showed an inverse association between duration of the intervention and CBF (P =0.02) but no influence of age, BMI or dose (P < 0.05). Nitrate and nitrite supplementation did not modify CBF or CF. Further trials employing larger samples sizes and interventions with longer duration are warranted.

Introduction

Cognitive impairment and dementia are global health challenges because of the costs associated with management and treatment, severity of symptoms for the affected individual and impact on patients' families, carers and communities (Wortmann, 2012). Furthermore, the prevalence of people diagnosed with dementia is rising at an alarming rate, with a recent report estimating that by 2050 the total number of individuals living with dementia worldwide will increase from 47 to 131 million (Prince et al., 2016). Therefore, effective interventions to prevent cognitive decline and dementia onset are a global research priority.

A major risk factor for cognitive decline is thought to be inadequate nitric oxide (NO) availability (de la Torre & Stefano, 2000; Toda et al., 2009). NO is a free radical soluble gasotransmitter with pleiotropic actions, of which several are integral to normal cognitive function (CF), including regional blood flow, immune-surveillance, metabolic efficiency, glucose homeostasis, and neurotransmission (Toda et al., 2009; Weitzberg & Lundberg, 2013). NO availability is determined by the activity of NO synthases (NOS), which are widely distributed across tissues in different isoforms (endothelial, inducible, neuronal) (Lundberg et al., 2008; Weitzberg & Lundberg, 2013). In cognitive decline, NO generation via these pathways

becomes dysregulated resulting in chronic hypo-perfusion, neurodegeneration and impaired cognitive ability (de la Torre & Stefano, 2000; Toda et al., 2009).

NO can also be produced by a distinct alternative pathway involving the conversion of nitrate into nitrite and NO via a series of reducing reactions (Lundberg et al., 2008; Zweier et al., 1995). Both nitrate and nitrite are present in a wide range of concentrations in a variety of foods with the higher content found in green leafy vegetables, beetroot, or meat products that have had nitrite salts added as preservatives (Lidder & Webb, 2013). In the past decade, it has emerged that increasing nitrate and nitrite ingestion may improve vascular and metabolic outcomes via increased generation of NO (Weitzberg & Lundberg, 2013). More recent evidence also indicates potentially beneficial effects of both compounds, administered as ionic salts or nitrate-rich food products, on cognition and brain metabolic and vascular health (Clifford et al., 2015; Gilchrist et al., 2014; Justice et al., 2015; Presley et al., 2011; Wightman et al., 2015). Such effects could be due to improved NO-mediated synaptic activity and/or as a consequence of increased cerebral blood flow (CBF) and thus a better coupling of blood flow to metabolism (Presley et al., 2011; Toda et al., 2009; Aamand et al., 2013).

Mechanistic support for the latter hypothesis in humans has been provided by Presley and colleagues (2011), who observed that a diet rich in nitrate-containing foods (e.g., green leafy vegetables) stimulated cerebral perfusion in the prefrontal cortex of elderly adults, the region of the brain associated with executive function, working memory, and other processes reliant on cognitive ability. However, subsequent studies measuring CBF, or directly measuring CF, after inorganic nitrate or nitrite ingestion have produced mixed findings, possibly because of the small size and diversity of study designs employed (Clifford et al., 2015; Kelly et al., 2013). Thus, despite the therapeutic potential, it remains unclear whether augmenting NO bioavailability with

either nitrite salts or nitrate-rich foods is an effective strategy for increasing CBF and/or mitigating cognitive deficits.

Consequently, we undertook a systematic review and meta-analysis of randomised clinical trials (RCTs) examining the efficacy of inorganic nitrate and nitrite supplementation on CBF and CF in adult participants with and without medical conditions. We set out to determine whether the ingestion of nitrite-salts or nitrate-rich foods (i.e., beetroot, spinach, rocket, lettuce, cabbage; Lidder & Webb, 2013) augments CBF and improves CF and to estimate effect sizes. We also examined whether test conditions (e.g., exercise vs. rest), age, body mass index (BMI), supplement dose, quality of the studies and intervention duration modified the effects of inorganic nitrate or nitrite on CBF and CF. These results will help to inform whether nitrate or nitrite supplementation holds promise as a relatively inexpensive strategy for augmenting CBF and combatting cognitive decline.

Methods

The present systematic review was conducted according to the Cochrane guidelines and it is reported according to PRISMA guidelines (Higgins & Green, 2011; Liberati et al., 2009). The protocol of the systematic review is available on request.

Literature search: Two databases (PubMed, Embase) were searched for articles from inception until May 2017. In addition, included reviews and eligible full text articles were searched manually to identify other suitable articles to be included in the systematic review. The following terms and keywords were entered and Boolean terms were used to increase sensitivity of the search strategy: nitrate, nitrite, beetroot, rocket, cabbage, lettuce, spinach, green leafy vegetables, cognition, brain, dementia, cerebral, memory, executive, attention, motor skills, blood flow,

vascular flow, perfusion. A summary of the specific search algorithms is reported in the **Online Supplementary Material (Box 1).**

Study selection: Titles and abstracts were screened using pre-defined eligibility criteria in accordance with the PICOS (population, intervention, comparator, outcome, study design) framework (Table S1 of the Online Supplementary Material) before retrieval of the full-text articles. The following inclusion criteria were used to assess the eligibility of articles for inclusion in this systematic review: 1) randomised controlled trials (no exclusion criteria were used for study design, or blinding); 2) trials recruiting adult participants (≥18 years) and no exclusion criteria were applied in relation to participants' health status: 3) trials based on nitrate or nitrite supplementation were included if they provide information on the type of nitrate salt (potassium or sodium), dose, formulation, frequency and route of administration. A list of .the inclusion and exclusion criteria is provided in the Online Supplementary Material (Box 2). Trials based on beet root juice supplementation or ingestion of nitrate-rich foods were included in the analyses if they provided information on the frequency and amount of nitrate-containing food provided; 4) trials reporting effects of nitrate or nitrite on global and domain-specific CF and CBF measured by different techniques including magnetic resonance imaging (MRI), ultrasound or near infrared spectroscopy (NIRS); 5) English-language restriction but not time restriction was applied in searching the databases; 6) Full text papers and abstracts were included (if they contained sufficient information to complete qualitative and quantitative analysis). Two investigators (TC, OS) independently evaluated the titles and abstracts to check eligibility for inclusion. If the reviewers agreed, each article was either excluded or moved to the next stage (full-text). If agreement was not achieved, the article was moved for evaluation after retrieval of the full-text. The selected full-texts were then reviewed to confirm their inclusion in the

systematic review. Disagreements were discussed with a third reviewer (MS) and resolved by consensus.

Data extraction: Relevant information was extracted and tabulated separately for CF and CBF. If information was not available from the full text, authors were contacted to obtain the relevant data.

Cognitive function: The following information was extracted independently by two investigators (AB, TC) from eligible articles: 1) authors and year of publication; 2) study characteristics (design, sample size); 3) participant characteristics (age, male/female ratio, health status and baseline values for BMI; 4) route, dose and duration of inorganic nitrate and nitrite supplementation; and 5) cognitive tests and exercise condition. Any disagreements in data extraction were resolved through discussion until consensus was reached.

Cerebral blood flow: Two independent reviewers (AB, MS) extracted relevant information from the eligible articles: 1) authors and year of publication; 2) study characteristics (design, sample size); 3) participant characteristics (age, male/female ratio, health status and baseline values for BMI, and 4) route, dose and duration of inorganic nitrate/ nitrite supplementation 5) method to assess cerebral blood flow (CBF) and testing conditions (i.e., exercise, mental stimulation). Any disagreements in data extraction were resolved through discussion until consensus was reached.

Quality Assessment: The modified Jadad score was applied to evaluate the risk of bias of the trials. Specific questions linked to randomisation procedure, blinding and description of dropout or attrition rates were used rank the quality of the trials (Jadad et al., 1996). Scores ranged from 0

to 5; a score less than 3 indicates a low quality trial where a score greater or equal to 3 indicates high quality trial.

Statistical Analysis

The primary outcomes of the meta-analysis were changes in CF and CBF after inorganic nitrate or nitrite supplementation. Random effect models were applied to address the heterogeneity related to differences in study design and application of different and concomitant methods for the evaluation of CF and CBF. In addition, some trials used several cognitive tests to assess domain-specific changes in CF and CBF, as shown in **Table 1 and 2**. This may lead to reduced independence of measurements and to consequential over-estimation of the effect size derived from the meta-analysis. These methodological aspects were taken into account into the analysis by averaging the standardised effect sizes for each trial with the aim of providing a more conservative estimate of the effect size. Forest plots were created to summarise and illustrate the individual and overall effects of inorganic nitrate and nitrite supplementation on CF and CBF. The meta-analysis was conducted using Comprehensive Meta-Analysis software (Biostat, Engelwood, New Jersey). Results are described as standardized mean differences (SMDs) and 95% confidence intervals (95%CI). If data were not available in the main text or in tables, figures were used to extract the information.

Sensitivity analyses were performed to investigate whether the effects of inorganic nitrate and nitrite supplementation on CF and CBF were influenced by testing conditions (i.e., exercise or mental stimulation). A random-effect meta-regression model was applied to examine the associations between effect sizes for CF and for CBF and age, BMI, dose of nitrate/nitrite supplementation, duration of the trial and Jadad score. Funnel plots and Egger's regression tests

were performed to evaluate the publication bias (Egger et al., 1997). Heterogeneity was assessed by using Cochrane Q statistic; P > 0.1 indicates significant heterogeneity. The I2 test was utilised to assess heterogeneity across trials where a value < 25% indicates low risk, 25-75% indicates moderate risk, and >75% indicates a high risk (Higgins et al., 2003).

Results

Search results

The screening process and the number of the studies included in the systematic review are described in **Figure 1**. The initial search of the two electronic databases produced 12865 articles which was reduced to 5387 after the deletion of duplicates. No relevant studies were found by manual search of relevant reviews and studies. After the first title and abstract selection phase, 23 full-text articles were identified for further assessment and, from these, 18 trials were included in the systematic review. Thirteen trials and nine trials were included in the meta-analysis to investigate effects of nitrate and nitrite supplementation on CF and CBF, respectively.

Cognitive Function

Studies characteristics: The trials included in the systematic review reported on a total of 297 participants with a median of 23 (range 10-48) participants per trial. The median age of the participants was 36 (range 21 – 73) years. The systematic review includes 2 parallel and 11 crossover trials and 12 of them were double-blind. Six of these studies included an exercise component as part of the protocol to evaluate the effects of dietary nitrate and nitrite on CF at

rest and during exercise. The large majority (12 of 13 studies) supplemented with nitrate or nitrate-rich foods; eleven trials used beetroot and one trial used spinach as sources of inorganic nitrate, and one study supplemented with sodium nitrite (see **Table 1**). As placebo, eight trials used nitrate-depleted beetroot juice (Kelly et al., 2013; Gilchrist et al., 2014; Lefferts et al., 2015; Rattray et al., 2015; Thompson et al., 2015; Thompson et al., 2016; Vanhatalo et al., 2016; Shannon et al., 2017), one studied employed nitrite-free capsules (Justice et al., 2015), two trials combined apple and blackcurrant juice (Thompson et al., 2014; Whitman et al., 2015) and one study did not report information on the control group (Bondonno et al., 2014).

Participant health status and intervention duration: Two trials included patients with type 2 diabetes (T2DM) (Gilchrist et al., 2014; Shepherd et al., 2015), four trials included middle-aged and older healthy participants (Kelly et al., 2013; Bondonno et al., 2014; Justice et al., 2015; Vanhatalo et al., 2016) and the remaining seven trials recruited young healthy participants (**Table 1).** The median BMI of the adults included in the trials was 24.6 kg/m^2 (range: 24.0 – 30.8 kg/m^2). The duration of interventions ranged from 90 minutes to 10 weeks but ten trials (out of 13) had a duration less than 7 days. For nitrate supplementation studies, the median dose of inorganic nitrate provided was 7.2 mmol/day (range: 2.9 – 12.8 mmol/day); the trial using nitrite supplemented with 2.4 mmol/day of sodium nitrite (Justice et al., 2015).

The greatest source of heterogeneity in the CF trials was the type of cognitive assessment with 23 different tests being reported. Three trials used a single CF test (Rattray et al., 2015; Thompson et al., 2015; Vanhatalo et al., 2016) whereas one trial employed eight different CF tests (Lefferts et al., 2015). A summary of the distribution of cognitive tests per trial is provided in **Table 1** whereas the frequency of application of each test across all the trials is summarised in **Figure S1** of the **Online Supplementary Material.**

Meta-analysis: Overall, inorganic nitrate or nitrite supplementation did not improve CF (SMD +0.06, 95% CI: -0.06, 0.18, P = 0.32) and we observed no significant heterogeneity between studies (I2 = 0%; P = 0.68) **(Figure 2).** However, the only study which supplemented healthy older individuals with inorganic nitrite for 10 weeks reported a significant improvement in CF (Justice et al., 2015). When stratified by inclusion of exercise testing in the protocols, there was no significant effect of inorganic nitrate supplementation in either the exercise (N=6, SMD +0.13, 95% CI: -0.05, 0.32, P = 0.16) or non-exercise (N=7, SMD +0.02, 95% CI: -0.15, 0.21, P = 0.76) trials. Meta-regression analysis did not reveal any significant association between CF effect size and age (β: -0.002, SE: 0.003, P = 0.33), BMI, (β: -0.02, SE: 0.02, P = 0.23), dose (β: 0.002, SE: 0.004, P = 0.63), study duration (β: 0.0005, SE: 0.0002, P = 0.07) or Jadad score (β: 0.09, SE: 0.18, P = 0.09) **(Table 3).**

Study Quality and Publication bias: The quality of the trials ranged from 2 to 5 (median: 3) on the Jadad score and only one study had a score < 3 (Bondonno et al., 2014), indicating the overall high quality of the trials **(Table 1).** Visual inspection of the Funnel Plot revealed a study with a large positive effect size and the presence of publication bias was also confirmed by the Egger's Regression test (p=0.01; **Figure S3** of the **Online Supplementary Material**). Exclusion of the study (Justice et al., 2015) with the largest positive effect size removed the publication bias (N=12, Egger's test, P=0.13).

Resting and Stimulated CBF

Studies characteristics: Nine trials assessed changes in CBF in resting conditions and included a total of 163 participants (sample size range: 10 – 40); the overall median age of the participants was 22 years (range 20 – 70). Five of these studies also assessed CBF under stimulated

conditions (i.e., exercise (Bond et al., 2013; Curry et al., 2016; Lefferts et al., 2016; Thompson et al, 2014), or mental stimulation (Wightman et al., 2015)).

One study employed a parallel study design (Whitman et al., 2015) whereas all remaining eight trials used a cross-over design (Aamand et al., 2013; Bond et al., 2013; Chirinos et al., 2017; Curry et al., 2016; Lefferts et al., 2016; Presley et al., 2011; Rattray et al., 2015; Thompson et al, 2014) **(Table 2).** Most studies (seven) used beetroot juice as a source of inorganic nitrate but high nitrate foods or sodium nitrite were also used in some studies **(Table 2).**

Cerebral blood flow tests: Four studies reported the effect of inorganic nitrate supplementation on middle cerebral artery blood flow velocity (MCAV) (Aamand et al., 2013; Curry et al., 2016; Lefferts et al., 2016; Rattray et al., 2015) and two reported the effect of inorganic nitrate on CBF measured by arterial spin labelling (Presley et al., 2011; Aamand et al., 2013). Additional measurements used to assess CBF included Near Infrared Spectroscopy (Thompson et al, 2014; Whitman et al., 2015), cerebrovascular resistance index by Transcranial Doppler Ultrasonography (Bond et al. 2013) and evaluation of changes in Carotid Characteristic Impedance, Carotid Cross-Sectional Area and Carotid Bed Vascular Resistance (Chirinos et al., 2017). The frequency of application of each method across all the trials is summarised in **Figure S2** of the **Online Supplementary Material.**

Participant health status and intervention duration: Eight trials recruited healthy individuals and one trial recruited patients with heart failure (Chirinos et al., 2017) **(Table 2).** The duration of the inorganic nitrate supplementation ranged from 3 hours to 3 days. The dose of inorganic nitrate ranged from 5.5 to 24 mmol/day (median dose: 9.8 mmol/day).

Meta-analysis: Overall, inorganic nitrate did not improve CBF under either resting (SMD +0.14, 95% CI: -0.13, 0.41, P = 0.31), or under stimulated conditions (SMD +0.23, 95% CI: -0.11, 0.56, P = 0.19). We observed moderate heterogeneity between studies testing the effect of inorganic nitrate on CBF at rest and stimulated conditions (I2 = 56.7%; P = 0.01; I2 = 44.1 %; P = 0.12, respectively) **(Figure 3).** Meta-regression analysis produced no evidence for significant associations of resting CBF effect size with age (β: 0.001, SE: 0.006, P = 0.98), BMI (β: 0.016, SE: 0.019, P = 0.41), dose (β: -0.01, SE: 0.019, P = 0.58), or Jadad score (β: 0.03, SE: 0.13, P = 0.79). However, there was a significant negative association between CBF effect size and study duration (β: -0.001, SE: 0.0006, P = 0.02) **(Table 3).**

Study Quality and Publication bias: The quality of the trials ranged from 2 to 4 (median: 2) according to the Jadad score. On this scoring system, *4* studies showed a score ≥ 3 (Chirinos et al., 2017; Lefferts et al., 2015; Thompson et al, 2014; Wighman et al., 2015) **(Table 2).** We could not assess the quality of one study (Ratary et al., 2015), as it was an abstract. Visual inspection of the Funnel Plot revealed no evidence of publication bias and this was confirmed by the Egger's Regression test for both resting (p=0.43) and stimulated |(p=0.58) CBF; **Figure S4** and **S5** of the **Online Supplementary Material).**

Discussion

Our meta-analysis revealed that inorganic nitrate or nitrite supplementation was not associated with improved CF or increased CBF. The combined standardized mean difference (placebo vs. intervention) was +0.06 for CF and +0.14 and +0.23 for CBF at rest and in simulated conditions, respectively. These findings were not influenced by whether the tests were performed at rest, during exercise or with mental stimulation, by the age or health status of the participants or by

the dose of inorganic nitrate or nitrite. Overall, the studies had small sample sizes and were of short duration, making it difficult to draw definitive conclusions about the efficacy of inorganic nitrate or nitrite in modulating CF and CBF.

The quality of the studies assessing CF was generally high; all studies employed a randomized design, used appropriate interventions, and in all but one of these studies (Bondonno et al., 2014) the intervention agent was provided in a double-blind fashion. Similarly, with the exception of one study, those assessing effects on CBF were all randomized, crossover trials. However, only 6 of the 9 trials were double-blind so that (along with other factors) meant they were generally of lower quality than those assessing effects on CF **(Table 2).** The overall utility of all the included trials was severely limited by the small sample sizes. Indeed, only 2 of the 21 studies reported that they had conducted an *a priori* power analysis to determine if they had an adequate sample size to detect a treatment effect for CF or CBF. One of these studies (Bondonno et al., 2014), suggested that at 80% power, 30 participants was sufficient to detect subtle treatment effects (e.g., 27 ms in simple reaction time) in various cognitive tasks. Given that the median sample size in the studies that assessed CF was only 23, it would be reasonable to assume that many of the studies were not adequately powered to detect potential effects of the nitrate or nitrite interventions. and that the risk of type 2 errors was high. In view of this, it is vitally important that future studies include larger sample sizes and ensure they are sufficiently powered to detect anticipated nitrate/nitrite-induced changes in CBF or CF.

The participants in most studies were of normal BMI, male, healthy, and not suffering from a cognitive-related disease. Of the two studies that examined effects of inorganic nitrate or nitrite on CF in a non-healthy cohort (T2DM patients), one observed improvements following the intervention (Gilchrist et al., 2014) and one did not (Shepherd et al., 2015). All remaining 11

trials that investigated effects of nitrate/ nitrite supplementation on CF were performed in participants with a BMI ≤25 kg·m^2. Because obesity is associated with impaired NO availability, it could be argued that individuals with a BMI ≥30 kg·m^2 might be more responsive to nitrate or nitrite induced vascular or metabolic effects (Ashor et al., 2016). Conversely, consequent to their greater body mass, it is possible that obese individuals will require a larger absolute nitrate/nitrite dose to manifest meaningful physiological changes. Prescribing a nitrate dose relative to body mass could help ameliorate this issue. Future studies should compare the effects of nitrate or nitrite supplementation on CF in both normal weight and obese individuals. As for studies that assessed effects on CF, only one study assessed effects of inorganic nitrate supplementation on CBF in non-healthy, obese participants. Chirinos and colleagues (2017) examined the effects of nitrate-rich beetroot juice in heart failure patients, and observed no significant changes in carotid artery hemodynamics. Arguably, older individuals suffering from a disease — especially a diagnosed cognitive disorder — are more likely to benefit from an intervention attempting to re-establish a dysfunctional pathway than young, healthy individuals, in whom NO availability is less likely to be impaired. Thus, it would seem prudent that future research prioritizes studying the effects of inorganic nitrate and nitrite supplementation on CF and CBF with older individuals with some cognitive dysfunction e.g. mild cognitive impairment or subjective memory complaints. In addition, few studies were carried out using female participants. Although there is no strong *a priori* rationale to anticipate that the impact of such supplementation would differ by sex, future studies should address potential effects in women.

Our meta-regression showed that the duration of the nitrate or nitrite supplementation had a modest influence on CBF. More specifically, the longer the duration of the supplementation, the smaller was the improvement in CBF. However, this observation should be interpreted with

caution because the majority of the trials had a very short duration. Of the nine studies assessing effects on CBF, only two provided the supplement for >150 min pre-assessment — and both displayed positive effects. The first, by Presley et al., (2011), was a randomized crossover trial in which healthy older adults received either a low nitrate or high nitrate diet for 2 days prior to measurements of cerebral perfusion using magnetic resonance imaging (MRI). Those in the high nitrate diet (12.6 mmol/day) group had a substantial and preferential increase in frontal cortex perfusion compared to those in the low nitrate diet group (0.9 mmol/day). The other study, by Aamand and colleagues, (2013) found that 3 days of sodium nitrate (vs. nitrate-free saline) decreased the haemodynamic lag of the blood oxygenation level dependent (BOLD) response in the visual cortex of healthy, young males **(Table 2)**. However, CBF, as measured by MRI, was unchanged. Clearly, more studies with longer supplementation periods are required before we can establish whether duration moderates the efficacy of nitrate/nitrite on CBF.

Most of the studies provided nitrate in the form of beetroot juice or nitrate-rich foods such as green leafy vegetables. Given only two studies assessed the effects of nitrate/ nitrite salts on CF or CBF, it was not possible to examine whether the vehicle for nitrate delivery (i.e., nitrate salts or nitrate-rich vegetable products) influenced the efficacy of supplementation. Interestingly, however, compared with nitrate salts, recent studies have reported greater effects of nitrate-rich vegetable products on blood pressure (Jonvik et al., 2016), the oxygen cost of exercise (Flueck et al., 2015), and post-exercise recovery (Clifford et al., 2017). This suggests possible additive or synergistic effects between nitrate and other plant-based compounds. Indeed, several plant-based compounds other than nitrate have potential benefits on CF and CBF (Ide et al., 2014; Desideri et al., 2012; Macready et al., 2009). These compounds include polyphenols, such as catechins, anthocyanins, and other flavonoids, and carotenoids (Macready et al. 2009; Gómez-Pinilla,

2008) that are purported, at least in part, to exert their beneficial effects on CBF and CF through NO-dependent mechanisms, namely increased vasodilative effects (Sokolov et al., 2013). To our knowledge, there is no evidence to suggest that beetroot, the main vehicle used in the RCTs included in this analysis, contains high quantities of the polyphenolic compounds showing potential for cognitive modulation. Indeed, the most abundant bioactive compound in beetroot, other than nitrate, is betanin and, to date, its effects on cognitive function are unknown. Notwithstanding, we acknowledge that the current evidence makes it impossible to differentiate the effects of nitrate/nitrite salts and nitrate-rich plants on cognitive function, the latter of which contains additional bioactive compounds. The independent effects of the bioactive compounds and the nitrate/nitrite in these foods is an important question for future research.

Our study also has a number of other limitations. Firstly, because such a wide range of assessments and methods were used to evaluate CF, several of which were domain-specific (e.g., reaction time vs. working memory), pooling the average effect size for all tests overlooks potential changes for isolated tests. This is illustrated by the fact that when each cognitive test was modelled as an independent outcome in the meta-analysis, nitrate supplementation showed a modest benefit for CF (data not shown). Nonetheless, this latter finding, in which all tests are considered independently, can overestimate the effect size; hence, to provide a more conservative estimate, we chose to use the average effect size from each study as our main outcome measure. Secondly, we observed moderate heterogeneity between studies for CBF, likely because of the wide variability in participant age and health status, CBF measures used, and the dose and duration of the nitrate/nitrite interventions used in each study. As outlined in a recent commentary (Barnard et al., 2017), heterogeneity between studies may disguise the benefits observed in single, well-controlled studies that, under specific conditions (e.g., dose,

duration, population) demonstrated *real* effects. This possibility needs to be taken into consideration when interpreting our findings.

Conclusions: In conclusion, there is no robust evidence that inorganic nitrate or nitrate supplementation influences CBF or CF. However, these findings might not be generalizable to older people, those with higher adiposity or and those with reduced cognitive ability, all of the included studies were performed in individuals <75 years old. In addition, all available trials were characterized by small sample sizes and short intervention durations and, thus, most of the studies may not have been designed optimally to observe any potential benefits. Consequently, the main conclusion of this study is that there is insufficient evidence to know whether supplemental inorganic nitrate or nitrite could improve CF or enhance CBF. Given the interest in use of non-pharmacological approaches for maintenance and improvement of cognitive function during ageing and the mechanistic rational for potential benefits of enhanced NO availability, further well-controlled and sufficiently powered trials, especially in more at-risk populations, with longer duration of nitrate/ nitrate supplementation, need to be conducted.

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Figure Legends

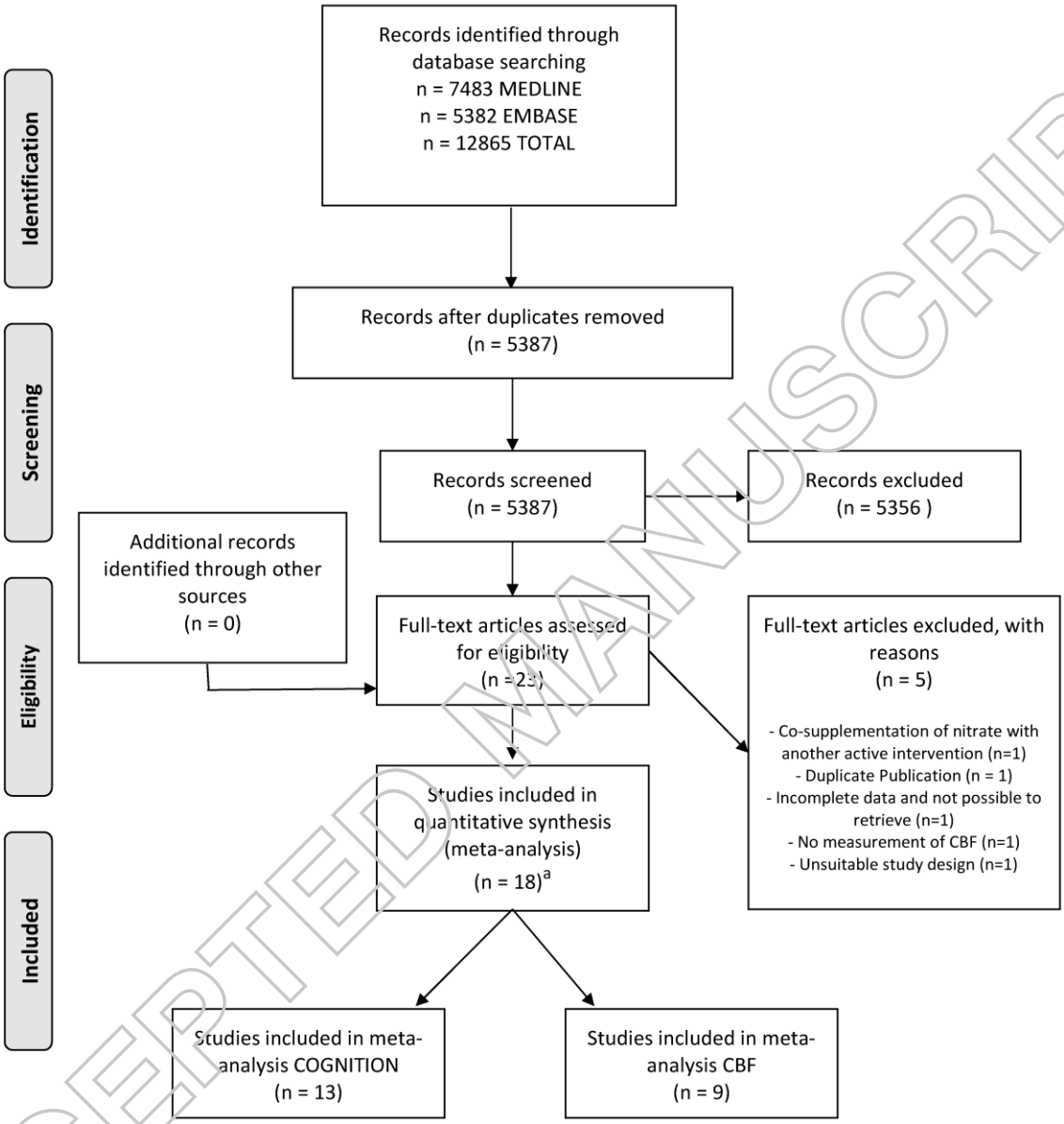

Figure 1: Flow diagram of the process used in selection of the randomised controlled trials included in this systematic review and meta-analysis

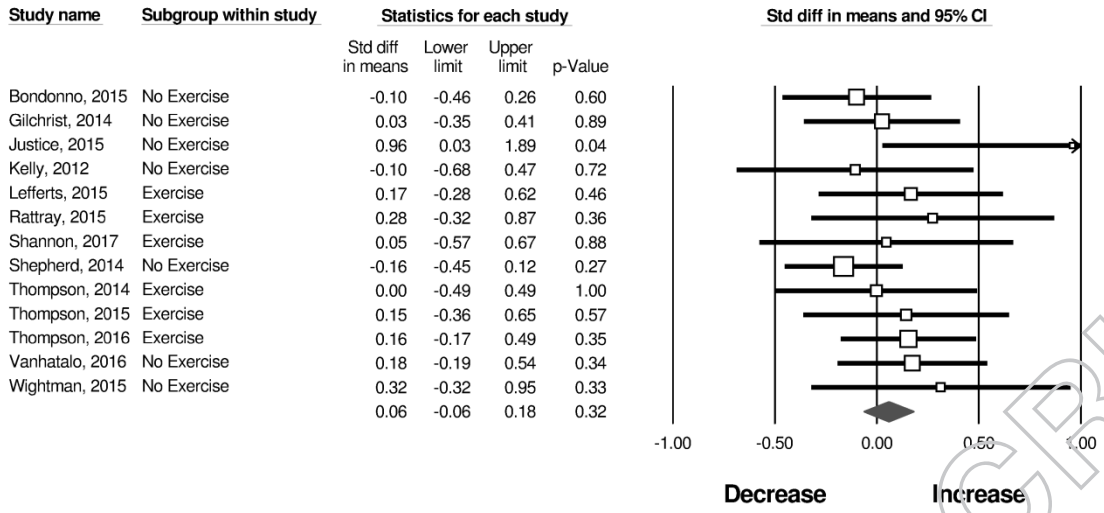

Figure 2: Forest plots showing the effect of dietary nitrate and nitrite supplementation on cognitive function.

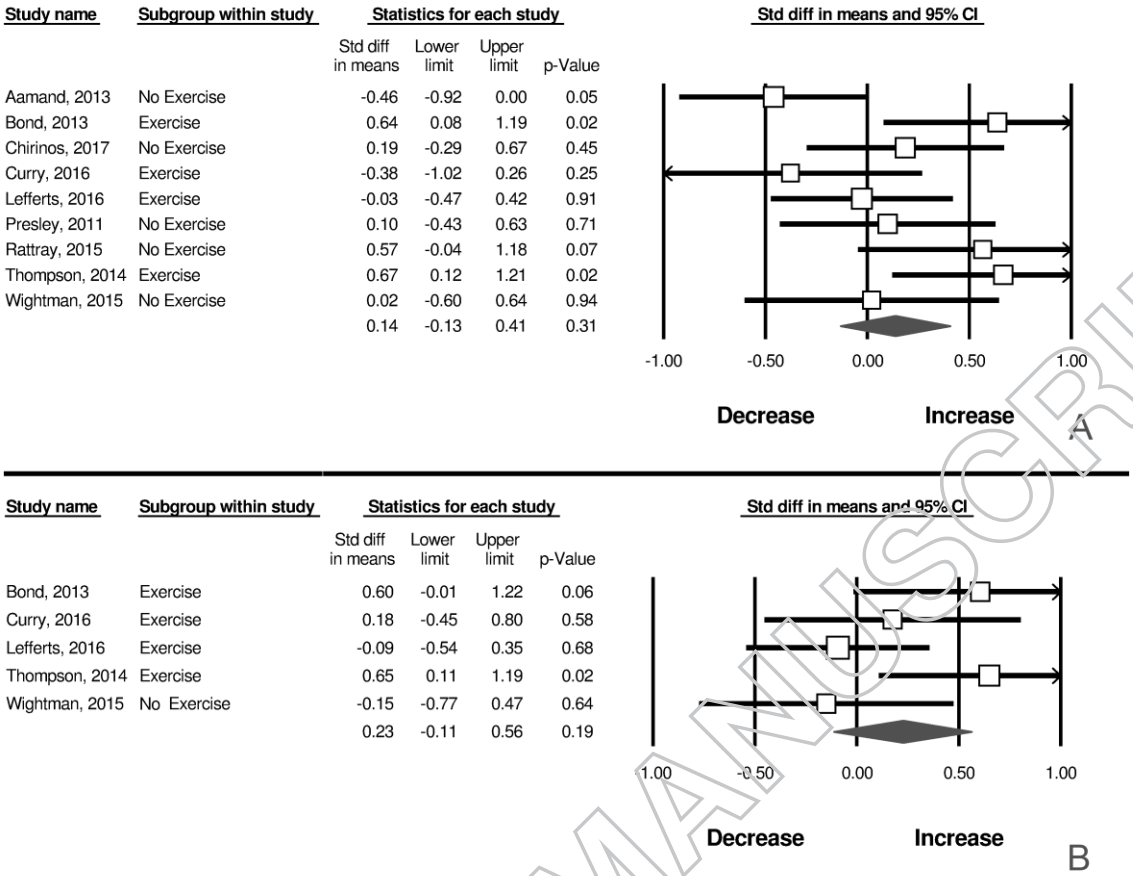

Figure 3: Forest plots showing the effect of dietary nitrate and nitrite supplementation on cognitive function, cerebral blood flow at rest (A) and in stimulated conditions (B).

Table 1: Characteristics of the studies included in the systematic review and meta-analysis of the effects of dietary nitrate/nitrite on cognitive function

Author (year)	Country	Study Design	Sample Size	Health Status	Age (years)	Males	Nitrate Dose (mmol/day)	Type of Intervention	Placebo	Duration of intervention	Baseline BMI (Kg/m^2)	Cognitive Tests	Exercise Testing?	Jadad Score
Bondonno, 2014	Australia	CO, R, UB	30	Healthy Middle-Aged	47.3	6	2.9	SP	-	150 min	23.6	SRT, DV, CRT, SM, NWM, DWR	NO	2
Gilchrist, 2014	UK	DB, CO, R, Pl	27	T2DM	67.2	18	7.5	BJ	ND-BJ	14 days	30.8	SRT, SM, RVIP, DRT, SPM	NO	3
Justice, 2015	USA	DB, P, Pl, R	30	Healthy Older	62	16	1.2/2.4	SN	NF-C	10 weeks	24.9	TMT-A TMT-B	NO	4
Kelly, 2013	UK	DB, CO, R, Pl	12	Healthy Older	64	6	9.6	BJ	ND-BJ	3 days	24.1	RVIP, SS, NR	NO	3
Lefferts, 2015	USA	DB, CO, R, Pl	20	Healthy, Young	23	20	6.5-7.0	BJ	ND-BJ	120 min	24.6	MR, ER, DV, AST, CRT, MZ, CPT, GNG	YES	3
Rattray, 2015[a]	Australia	DB, CO, R, Pl	12	Healthy, Young	-	-	12	BJ	ND-BJ	120 min	-	CST	YES	-
Shannon, 2017	UK	DB, CO, R, Pl	10	Healthy, Young	23	10	12.5	BJ	ND-BJ	175 min	23.9	SST, AST, RVIP	YES	3
Shepherd, 2015[a]	UK	DB, CO, R, Pl	48	T2DM	63.3	35	6.4	BJ	ND-BJ	4 days	30.1	SRT, SM, CST	NO	
Thompson, 2015	UK	DB, CO, R, Pl	16	Healthy, Young	24	16	12.8	BJ	ND-BJ	7 days	24.6	CST, DRT	YES	3
Thompson, 2014	UK	DB, CO, R, Pl	16	Healthy, Young	24	16	5	BJ	BCJ+AJ	90 min	24.1	RVIP, CST	YES	3
Thompson, 2016	UK	DB, CO, R, Pl	36	Healthy, Young	24	36	6.4	BJ	ND-BJ	5 days	24.6	CST	YES	3
Vanhatalo, 2016[a]	UK	DB, CO, R, Pl	30	Healthy Older	73	10	12	BJ	ND-BJ	10 days	25	RVIP	NO	-
Wightman, 2015	UK	P, DB, R, Pl	40	Healthy, Young	21	12	5.5	BJ	BCJ+AJ	90 min	24	SS, RVIP, MFT	NO	3

BCJ+AJ, blackcurrant cordial JUICE and apple juice; BMI, body mass index; CAD, coronary artery diseases; CO, crossover; Conc, concentration; DB, double-blind; NF-C, nitrite free capsules; P, Parallel; Pl, placebo-controlled; R, Randomized SB, single-blind; SN, Sodium Nitrite; SP, spinach T2DM, type 2 diabetes; UB, non-blind. SS, Serial Subtractions, RVIP, Rapid Visual Information Processing; MFT, Mental Fatigue Test; CST, Colour Stroop Test; SRT, Simple Reaction Time; SM, Shape Memory; DRT, Decision Reaction Time; SM, Spatial Memory ; DV, Digit Vigilance ; CRT, Choice Reaction Time; NWM, Numeric Working Memory; DWR, Delayed Work Recognition ; AST, Attention Switching Task; SST, Spatial Span Task; TMT-A, Trail Making Tests A; TMT-B, Trail Making Test-B; NR, Number Recall; MR, memory recognition; ER, Emotion Recognition; VS-1, Visual Interference; VB-1, Verbal Interference; MZ, Maze, CPT, Continuous Performance Test; GNG, Go/No-Go. [a] These are abstracts and the quality assessment was not performed.

Table 2: Characteristics of the studies included in the systematic review and meta-analysis of the effects of dietary nitrate/nitrite on cerebral blood flow

Author (year)	Country	Study Design	Sample Size	Health Status	Age (years)	Males	Nitrate Dose (mmol/day)	Type of Intervention	Placebo	Duration of intervention	Baseline BMI (Kg/m^2)	CBF Assessment	Exercise Testing?	Effect at resting	Effect in stimulated conditions	Jadad Score
Aamand, 2013	Denmark	DB, Pl, ,CO, R	20	Healthy, Young	25	20	7.7	SNA	NF-S	3 days	-	ASL	NO	No change	-	2
Bond, 2013	USA	Pl, CO, R	12	Healthy, Young	20	-	5-6	BJ	OJ	120 min	24.4	CVRI, MCAV	YES	Positive	Positive	1
Chirinos, 2017	USA	DB, CO, R, Pl	17	HFpEF	65	14	12.9	BJ	ND-BJ	150 min	34.4	CCID, CCSA, CBVRD	NO	Positive	-	4
Curry, 2016	USA	Pl, CO, R	10	Healthy, Young	20	-	24.2	BJ	OJ	120 min	23.5	MCAV	YES	Positive	Positive	1
Lefferts, 2015	USA	DB, CO, R, Pl	20	Healthy, Young	23	20	6.5-7.0	BJ	ND-BJ	120 min	24.6	MCAV	YES	No change	No change	3
Presley, 2011	USA	R,CO	16	Healthy, Old	≥ 70	NR	12.4	High nitrate diet	Low nitrate diet	2 days	-	ASL	NO	Positive (regional cerebral perfusion)	-	2
Rattray, 2015[a]	Australia	DB, CO, R, Pl	12	Healthy, Young	-	-	13	BJ	ND-BJ	120 min	-	MCAV	YES	Positive	-	-
Thompson, 2014	UK	DB, CO, R, Pl	16	Healthy, Young	24	16	5	BJ	BCJ + AJ	90 min	24.1	NIRS	YES	Positive	Positive	3
Wightman, 2015	UK	P, DB, R, Pl	40	Healthy, Young	21	12	5.5	BJ	BCJ + AJ	90 min	24	NIRS	NO	Positive	Negative	3

BCJ+AJ, blackcurrant cordial JUICE and apple juice; OJ, Orange juice; SN, Sodium Nitrate; NF-S, Nitrate free solution; BJ, Beetroot juice; ND-BJ, Nitrate depleted beetroot juice BMI, body mass index; CO, crossover; DB, double-blind; NF-C, nitrite free capsules; P, Parallel; Pl, placebo-controlled; R, Randomized SB, single-blind; ASL, Arterial spin labelling; CVRI, Cerebrovascular resistance index; SBP, Systolic blood pressure; TVR, Total vascular resistance; MCAV, Middle cerebral artery blood flow velocity; HFpEF, Heart failure preserved left ventricular ejection fraction; CCID, Carotid characteristic impedance, dynes; CCSA, Carotid cross-sectional area; CBVRD, Carotid bed vascular resistance, dynes. [a] This is an abstract and the quality assessment was not performed.

Table 3: Meta-regression analysis to evaluate whether age, BMI, dose of nitrate and duration of the intervention modified the effects of nitrate/ nitrite supplementation on cognitive and cerebral blood flow

	Slope (β)	**SE**	**Q (df)**	***P***
Cognitive function (n= 13)				
Age (years)	-0.002	0.003	0.92 (1)	0.33
BMI (kg/m^2)	-0.02	0.02	1.38 (1)	0.23
Dose (mg/day)	0.002	0.004	0.22 (1)	0.63
Duration (hours)	0.0005	0.0002	3.20 (1)	0.07
Jadad	0.30	0.18	2.85 (1)	0.09
Resting CBF (n= 9)				
Age (years)	0.001	0.006	0.09 (1)	0.98
BMI (kg/m^2)	0.02	0.032	0.58 (1)	0.45
Dose (mg/day)	-0.02	0.023	0.43(1)	0.51
Duration (hours)	-0.001	0.0006	5.07 (1)	0.02
Jadad	0.03	0.13	0.06 (1)	0.79

BMI, Body Mass Index; SE, standard error; CBF, cerebral blood flow.